Frog Skin Opioid Peptides: A Case for Environmental Mimicry

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Round the caldron go;
In the poison'd entrails throw.—
Toad, that under cold stone,
Days and nights hast thirty-one
Swelter'd venom sleeping got,
Boil thou first i' the charmed pot!
                    Macbeth (act IV, scene 1)

Shakespeare's witches realized that potions contained marvelous concoctions of ingredients (some obviously unattainable and mythical in nature), several of which could alone elicit the coveted effect. This celebrated soliloquy reflects a unique knowledge of hereditary folklore that particular parts of animals and plants affect human behavior (beliefs that continue in vogue even as the twentieth century comes to a close). In a sense, this witches' brew was a cocktail of unknown chemical composition that could mimic and interfere with human pharmacological responses (agonists or antagonists that activate or block pharmacological receptors leading to changes at a physiological level, such as stimulation or depression). Medicinal chemistry is a recent outgrowth of our historical quest to define an active component(s) from a host of biological sources— plants, invertebrates, and vertebrates alike (1–5)—much like modern chemistry arose from alchemy.

Environmental mimicry is a "copycat theme" (6) whereby organisms evolved to resemble something innocuous or poisonous as a defense against predation. Mimicry can also be considered "adaptive convergence" among different species living under similar environmental conditions, such as adaptation of oceanic vertebrates (past and present) with streamlined bodies and paddlelike limbs or fins (7,8) and antigenic convergence among phylogenetically diverse parasitic helminths in vertebrate hosts (9). In a broader sense, mimicry includes coloration (protective, obliterative, warning) and "protective resemblance," chemical resistance, and antigenic determinants (9). The classic example that we associate with environmental mimicry is "Müllerian mimicry"

(8), a well-known phenomenon amongst lepidopterans; for example, moths that take on the coloration and pattern of the bark on which they alight ("industrial melanism" is one such contemporary case) and the deliciously edible Viceroy butterfly that adopts the wing pattern of the unpalatable Monarch butterfly. Aposematism (same or a closely similar pattern shared by two or more species) is encountered frequently throughout the animal kingdom, and several examples illustrate this phenomenon. Among birds, three distinct species of African birds (the flycatcher, cuckoo-shrike, and tit) resemble the plumage of the aggressive drongos, while in New Guinea several species of pitohui and a female of an unrelated species (a bird of paradise) take on the colored plumage of the hooded pitohui (5) which contains the toxic homobatrachotoxin (a compound 500 times more potent than strychnine!) (10). Tropical and neotropical amphibians exhibit an abundance of mimicry in their striking color combinations, many of which are extremely poisonous (3). Reptilian mimicry is also evident in the coloration and similarity of the banding patterns between the innocuous king snake and the deadly coral snake. The semblance of an Australian seahorse to its coral habitat affords protection from predators.

Mimicry also occurs in distinct ways in other species, most notably among insects: the semblance of stick insects to dried twigs, moth larvae to dried leaves, and leaf insects to their habitat; some moths, beetles, and flies have even "copied" the physical appearance of various species of predatory wasps. A variety of plants, in their bid for insect pollinators, mimic not only the reproductive apparatus of other nonrelated species, but also physically resemble insects (such as bees) to trick them into mating with the plant in order to cover them with pollen. Other plants, in the Judean desert for example, acquire the red color that attracts scarab beetles who, during mating on the flower, carry away nearly 20 times more pollen than bees.

Naturally occurring environmental substances often mimic endogenous substances found in mammals and are capable of interacting with specific proteins, such as receptors, with a high degree of fidelity and selectivity. Narcotic alkaloids and amphibian skin secretions, introduced into human society through close association with plants and animals through folk medicine and religious divination practices, were incorporated into the armamentarium of the early pharmacopoeia. These skin secretions contain a myriad of potent bioactive substances, including alkaloids, biogenic amines, peptides, enzymes, mucus, and toxins (noxious compounds notwithstanding); each class exhibits a broad range of characteristic properties. One specific group of peptides, the opioids, containing the dermorphins (dermal morphinelike substances) and the deltorphins (δ-selective opioids), display remarkable analgesic properties and include an amino acid with the rare (in a mammalian context) D-enantiomer in lieu of the normal L-isomer. Synthesis of numerous stereospecific analogues and conformational analyses of these peptides provided essential insights into the tertiary composition and microenvironment of the receptor "pocket" and the optimal interactions between receptor and ligand that trigger a biological response; new advances in the synthesis and receptor-binding properties of the deltorphins are discussed in detail. These receptor-specific opioid peptides act as more than mimics of endogenous opioids: their high selectivity for either the μ or δ receptor makes them formidable environmentally derived agents in the search for new antagonists for treating opiate addiction and in the treatment of a wide variety of human disorders. *Key words:* amphibians, deltorphin, dermorphin, evolution, mimicry, molecular modeling, opiate addiction, opioid peptides, peptide synthesis. *Environ Health Perspect* 102:648–654 (1994)

This topic is not only fascinating to evolutionary biologists and ecologists, but also to biochemists, molecular biologists, and toxicologists (*1*). When examined at the molecular level, mimicry illustrates an even more bewildering diversity and bizarre theme of adaptation to a microenvironment (*6*). The synthesis and secretion of a bevy of chemicals serve a variety of purposes: attractants, for instance, in fungi or "fungal pseudoflowers," which reproduce the taste and odor of flowers to attract insects as vectors in their transmission (*6*); repellents, such as the toxic compounds associated with the monarch butterfly, hooded pitohui, and other organisms; camouflage, as beautifully seen in the complete correspondence, synthesis, and chemical identity of larval hydrocarbons of the fly *Microdon* to lull its obligate ant host/prey without setting off alarm bells (*2*); and defense, as depicted by the secretion of lethally toxic compounds by certain amphibians onto their skin in combination with vivid skin coloration (*1,3*), thoroughly illustrated among arthropods (*11*) and some plants (*12*). These examples merely represent some poignant illustrations of the use of environmental chemicals, a phenomenon aptly coined "chemical ecology" (*5*), to mimic natural products found in one species to bring about changes in other nonrelated species. This implies *de jure* that these extraneous substances interact with endogenous receptor molecules to initiate a chain of intracellular signaling events that elicit definable pharmacological, biochemical, and physiological responses. In fact, this concept of structure–activity relationships was elegantly addressed by investigators over the past two centuries who initially studied the pharmacological properties of plant alkaloids (*13–15*).

For millennia, human populations throughout the world have turned to plant materials (undoubtedly arising in the search for food) and amphibian secretions (*1*) to supply medicinal remedies and unctions to counter pain (analgesia) and diarrhea (antiperistalsis), to produce euphoria and a psychological sense of well-being, or to induce religious hallucinations and divination. For example, the Sumerians and the early dynastic Egyptians, 6,000 and 4,000 years ago, respectively, used extracts of *Papaver somniferum* in the treatment of pain and diarrhea (*16*).

Amphibian skins, depending on the genera and species, synthesize and secrete an amazing diversity of compounds. Skin secretions emanating from the granular glands contain five major classes of substances, but not all in any one species of amphibian (*1,3,4*): bufogenins (a family of heterocyclic substances that inhibit K^+/Na^+

ATPase) (*3*); alkaloids (including the major classes of dendrobatid alkaloids— batrachotoxins, histrionicotoxins, indolizidines, pumiliotoxin-A, and decahydroquinolines—and numerous minor classes— gephyrotoxins, 2,6-disubstituted piperidines, pyrrolidine alkaloids, pyridyl-piperidines, indole alkaloids, azatricyclododecenes, amidine alkaloids, epibatidine, samandarine alkaloids, morphine, tetrodotoxins, and a group of miscellaneous piperidine-based alkaloids), which collectively total more than 200 compounds primarily from toxic neotropic frogs of the genera *Dendrobates* and *Phyllobates* (*3*); biogenic amines (consist of three distinct groups, the indolealkylamines, imidazolealkylamines and phenylalkylamines, the latter of which includes common catecholamine neuromodulators adrenaline, noradrenaline, and dopamine) (*4*); peptides from 10 structurally and bioactively distinct families (totaling over 100 peptides, of which two-thirds exhibit a broad spectrum of potent biological activities in mammals and include tachykinins, bradykinins, caeruleins, bombesins, opioids, xenopsins, thyrotopin-releasing hormone, angiotensin, a heterogenous group of amphiphilic peptides with antimicrobial activities, and a collection of miscellaneous peptides and precursor fragments isolated from *Xenopus laevis*) (*1*); and proteins or proteinaceous components (such as enzymes involved in α-amidation and post-translational modification of peptides including endo- and exopeptidases and integumentary mucins, lectins, and toxins) (*1*).

The habitual use of mood-altering narcotics often leads to addiction and drug dependency (*17*), causes hypotension and respiratory depression, and readily establishes the premise for considering molecular mimicry: a potential requirement for highly specific complementary interactions between a ligand and its receptor involves stereochemical configuration, spatial conformation, and functional groups necessary for association (*18*). Establishing a function for newly discovered classes of opioid compounds[1] (*4*) provides the key to unraveling the mysteries of the opioid receptor.

In an evolutionary context, we might ask ourselves why we would expect to find environmental opioids to mimic endogenous substances and interact with mammalian receptor molecules. (Environmental estrogenic compounds that mimic mammalian steroids and bind with steroid

receptors are coming to the forefront in research on human disease and animal biology.) Have not mammals evolved in divergent directions for the past 230–255 million years from their amphibian ancestors or over an even greater time span from the experimental prototypes of life in the Cambrian and Proterozoic eras (two billion distant years past) that eventually gave way to our extant phyla? What could be the basis for molecular mimicry or the stability of receptor recognition mechanisms since the origin of protozoans, such as *Tetrahymena*, which has opioid receptors (*19,20*)? Could the protozoan δ receptor type be the archaic progenitor for μ and κ receptors based on the degree of sequence similarity among them by evolving (*21*) through various genetic mechanisms (mutation, gene duplication, genome rearrangement) (*1*)? We know that primitive neuronal systems (nerve networks) were not in place until the emergence of early metazoans; therefore we can surmise that opioid substances functioned differently than is assumed today. Hypothetically, an opioid receptor might have arisen in response to nutritional requirements (*22*), regulated ionic conductance (*23*), or affected reproduction long before a neuromotor system evolved per se. Interestingly, if one considers that "the underlying mechanism for neural activity is ionic" (*24*), then the ability of opioid peptides to modulate ion flux and adenyl cyclase activity suggests that the protozoan receptors might be preadapted to function in the control of ionic conductance when they became eventually located in a potential neuronal membrane through evolution.

Opioids and their receptors play vital roles in the overall homeostasis of mammalian physiology. The major attributes of opioid action in vertebrates can be briefly condensed as production of analgesia; modification of the secretion of circulating peptide hormones; alteration of body temperature; constriction of the pupil; depression of respiration and gastrointestinal function (acting as an emetic); involvement with the cough response; enhancement of peripheral vasodilation; and association with the immune system (*25*). Thus, in spite of the accumulation of mutational changes (the basis of hereditary variability), the maintenance of molecular mimicry in the opioid peptide-receptor relationship points to a highly stable system of interacting components.

One possible reason for the presence of a high concentration of opioids among the vast quantities of bioactive peptides secreted by the genus *Phyllomedusa* (*26*) could be an amphibian defense network (simply described as "overkill") (*1*): predators evolve means to circumvent "even the most

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[1] In this paper we wish to emphasize a differentiation in the use of the terms "opioid" and "opiate." The former denotes only peptides and the latter alkaloid (or nonpeptide) substances that exhibit morphinomimetic analgesia.

novel defense" (*3*); opioids represent a class of substances whose wide spectrum of activity provides another bastion of protection. The production and secretion of large amounts of bioactive substances is a further indicator of the amphibian environment: animals, such as frogs, who spend a portion of their time in aqueous environs will need higher concentrations of skin secretions due to dilution and persistent washing of their integuments by the water. By analogy, this would be like treating a microbial infection simultaneously with several general-acting antibiotics to ensure possible elimination of the causative agents. And like the excessive application of antibiotics, which leads to resistant organisms, amphibian predators evolved means to cope with the compounds in skin secretions and, in turn, the need for the production of more diverse compounds by amphibians.

Opioid Substances

The most commonly identifiable environmental chemicals of social concern—relative to opioid peptides—are the narcotic alkaloids, promulgated in the news media and governmental policy through the "war on drugs." Morphine (named after the Greek god of dreams, Morpheus), first crystallized in 1803 as a constituent of opium (*13*), triggers a biochemical response due to the complimentarity of its ring structure with the microenvironment (internal shape, ionic and hydrophobic milieu) of the receptor. The salient feature of the pharmacological effects of the opiate alkaloids, determined by the synthesis of a vast array of related drugs, is their high degree of stereoselectivity (*14*), analogous to that observed with the amphibian opioid peptides (*27*). In the case of the alkaloids, however, their affinities for the opioid receptors are relatively low—being orders of magnitude less than that of the endogenous enkephalins (*16,28*) or exogenous amphibian opioids (*29*).

The discovery of the enkephalins (*30*) and β-endorphin two decades ago (Table 1) explosively opened a new field in neuroscience. They generated interest in the relationship between opioids and the immune system (*31–34*) and continue to make headlines as β-endorphin is the supposed endogenous hormone released upon strenuous exercise (the body's elixir to adapt to physical strain and stress).

A seminal change (and a most fortuitous modification as discussed further in this commentary) in the concept of opioid structure occurred through the introduction of the D-enantiomer of alanine in place of glycine at the second position (Table 1) of a synthetic enkephalin peptide; this produced an analogue with pro-

Table 1. Sequences of naturally occurring opioid peptides

Peptide	Sequence[a]
Mammalian	
Enkephalins	
	Tyr-Gly-Gly-Phe-Leu
	Tyr-Gly-Gly-Phe-**Met**
	Tyr-Gly-Gly-Phe-**Met-Arg-Phe**
	Tyr-Gly-Gly-Phe-**Met-Arg-Gly-Leu**
β-Endorphin[b]	Tyr-Gly-Gly-Phe-**Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu**
Dynorphins[c]	
Dynorphin A	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln
Dynorphin B	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-**Gln-Phe-Lys-Val-Val-Thr-Arg-Ser-Gln-Glu-Asp-Pro-Asn-Ala-Tyr-Tyr-Glu-Glu-Leu-Phe-Asp-Val**
β-Neo-endorphin	Tyr-Gly-Gly-Phe-Leu-Arg-**Lys-Tyr-Pro**
Amphibian	
Dermorphins[d]	
	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH$_2$
	Tyr-D-Ala-Phe-Gly-Tyr-**Hyp**-Ser-NH$_2$
	Tyr-D-Ala-Phe-Gly-Tyr-**Hyp**-Ser
	Tyr-D-Ala-Phe-Gly-Tyr-Pro-**Lys**
	Tyr-D-Ala-Phe-**Trp**-Tyr-Pro-**Lys**
	Tyr-D-Ala-Phe-**Trp-Asn**
	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-**Gly-Glu-Ala**
	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-**Gly-Glu-Ala-Lys-Lys-Ile**
Deltorphins	
	Tyr-D-Met-Phe-His-Leu-Met-Asp-NH$_2$ (A)
	Tyr-D-**Ala**-Phe-**Glu-Val-Val-Gly**-NH$_2$ (B)
	Tyr-D-**Ala**-Phe-**Asp-Val-Val-Gly**-NH$_2$ (C)
	Tyr-D-**Leu**-Phe-**Ala-Asp-Val-Ala-Ser-Thr-Ile-Gly-Asp-Phe-Phe-His-Ser-Ile**-NH$_2$

[a]Amino acid residues in bold indicate differences with the parental peptide beginning each section. Unless specified, the C-termini contain a free carboxylic group (-COOH). All the mammalian peptides contain the identical N-terminal tetrapeptide sequence (Tyr-Gly-Gly-Phe), whereas the amphibian peptides are characterized by a D-enantiomer at position 2 of a common tripeptide sequence (Tyr-D-Xaa-Phe).
[b]The β-endorphin family consists of three peptides: α-, β-, and γ-endorphin, which are derived from the precursor protein, proopiomelanocortin. β-Endorphin contains the sequences of [Met^5]enkephalin (β-endorphin 1-5), α-endorphin (β-endorphin 1-16) and γ-endorphin (β-endorphin 1-17), the latter two represent either degradation products or processed fragments. Although β-endorphin and the dynorphins contain the [Met^5]- or [Leu^5]enkephalin sequence, neither one are the precursors for these opioids (*14,75*). The sequence of β-endorphin shown is that from camel.
[c]The structural similarity among the dynorphin family resides in residues 1-6. A commonly occurring dynorphin is the N-terminal octapeptide fragment, dynorphin A 1-8 (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile).
[d]The latter two dermorphin peptides were isolated, but represent fragments generated from the precursor before subsequent proteolytic cleavage and formation of the C-terminal amide from glycine by an enzymic α-amidation reaction (*1*). Smaller synthetic fragments of [D-Leu^2]deltorphin, sequences 1-10 and 1-7, weakly interact with δ and μ opioid receptors but are nonselective (*44*).

longed bioactivity (*35,36*) due to enhanced stability against proteolytic degradation (*35*). Literally, many hundreds of enkephalin analogues have been synthesized (*37*) based on this singularly important observation. Although the announcement in 1980 of a potent opioid peptide from amphibian skin (*38*), dermorphin, triggered considerable consternation (that a rare D-amino acid should be found in organisms other than bacteria, mold, and algae) (*1*), it should have been anticipated by the science community from the extensive body of literature on enkephalin analogues (*37*). By the end of the decade, another group of D-amino acid-containing opioid peptides was discovered from the same amphibian source (*38–40*)—the deltorphins, opioid peptides with the highest affinity and selectivity for δ opioid receptors (*40–43*), except the larger D-Leu-containing variant which is essentially devoid of both δ and μ activity

(*44*). Amphibian opioid peptides are not recognized by the mammalian κ receptor, the third distinct opioid receptor that binds the dynorphin family of opioid peptides (*37*) (Table 1). In spite of their limited size (a heptapeptide is small by most biochemical standards), it was proposed that the N- and C-terminal regions represented the common "message" and specific "address" domains, respectively (patterned after the model established for discrete sections of the adrenocorticotropin hormone molecule) (*45*).

An intriguing feature of the precursor proteins for opioid peptides, regardless of their biological source, is the existence of the coding for multiple bioactive peptides within a single genomic transcript (*16,39,40,46*). As a comparable example, the enkephalin prohormone precursor contains seven copies of bioactive peptide (*47*) (Fig. 1), which are excised by the action of specific proteolytic enzymes acting at

Preproenkephalin (267)

Preprodynorphin (256)

Preprodermorphin (197)
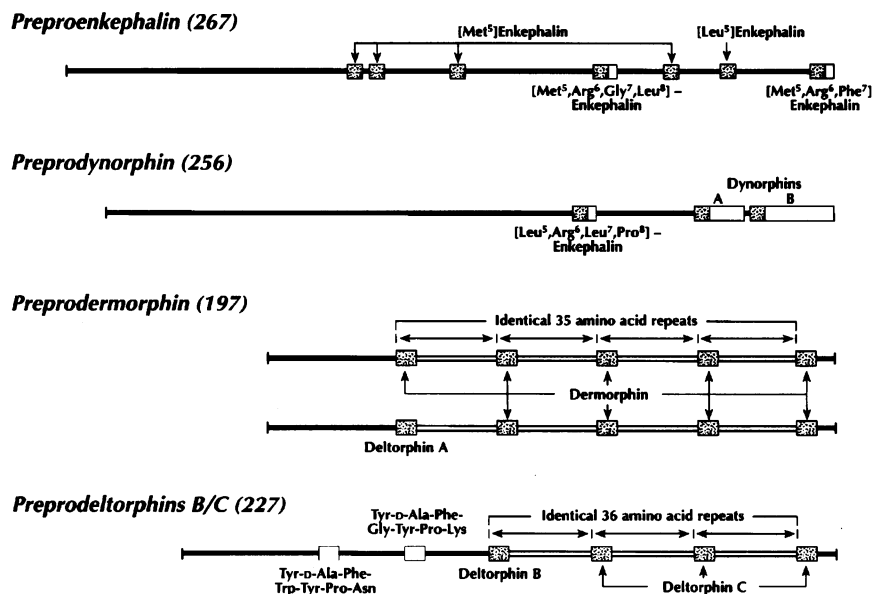

Preprodeltorphins B/C (227)

Figure 1. Diagrammatic structures of the preprohormones of enkephalin, β-neo-endorphin/dynorphin, dermorphin, deltorphin A/dermorphin, and deltorphins B and C/dermorphin analogues. The numbers in parentheses are the number of amino acid residues in the transcribed protein; the precursors are each drawn to scale. An interesting feature of the amphibian preprohormone is the greater use of the precursor protein; the tertiary structural features of the homologous repeat sequences may be involved in the inversion of the stereo configuration from L to D at the α-carbon of residue 2 of the bioactive peptide (*1*). The boxed regions indicate the presence of a potential opioid peptide; the stippled areas indicate prevalent bioactive peptides or sequences of direct homology for a given precursor. The solid line is the remainder of the precursor protein; in both the dermorphin and deltorphin prohormones, the narrow open bar denotes homologous sequences within the respective protein.
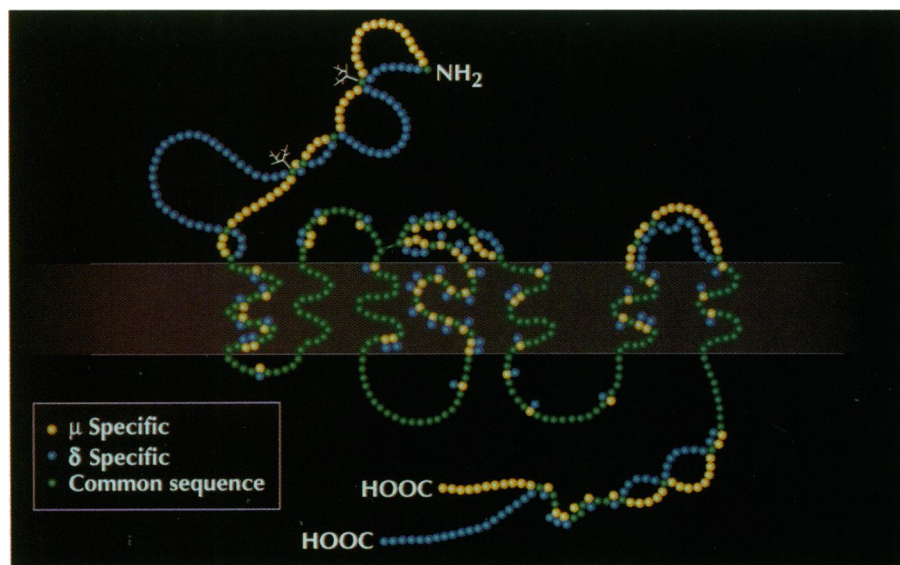

Figure 2. Structure of δ and μ opioid receptors. Each sphere in the chain represents an amino acid: the δ receptor-specific residues are blue, those for the μ receptor are yellow, and those common to both sequences are green; the branched structures represent presumed complex carbohydrate on threonine residues. The light shaded band depicts the cell membrane with the extracellular space above denoted by the common N-terminus (-NH$_2$) and the intracellular compartment below with the C-termini (-COOH).

D-enantiomer might represent either a novel post-translational modification in frog skin (*39*) or some other inversion mechanism (*1,49,50*). One cDNA clone of a dermorphin preprohormone contained the sequence of deltorphin A (*39*), while in the case of deltorphins B and C, the cDNA transcripts included one copy of deltorphin B and from zero to three copies of deltorphin C (*40*), as well as two new dermorphin-related peptides (Fig. 1). The multiplicity of bioactive peptides in a single precursor prohormone suggests the requirement of a temporal change—"fecundity"—that denotes either the high turnover of a labile product or the necessity for vast quantities of peptide to oversaturate the immediate vicinity of the amphibian with bioactive molecules. Remarkably, amphibian skins, for example, have the unique capacity to produce and secrete relatively high percentages of peptide in relation to the wet weight of their skins (*4,26,29*).

The presence of exogenous (environmental) opioid peptides clearly indicates that the mammalian opioid receptors retained a unique tertiary conformation during evolution in spite of differences in their sequences (*51–59*) (Fig. 2). The combination of sequence and receptor conformation enables them to selectively screen and bind peptide ligands which exhibit close structural similarity. A remarkable degree of sequence conservation in the opioid receptors is seen in the seven transmembrane regions (Fig. 2); the majority of differences reside, however, in the N-terminal domain and one extracellular loop (excluding the variability seen in the intracellular C-terminal portion) that could be responsible for the difference between binding of receptor-selective agonists and antagonists, or compounds that exhibit partial agonist and antagonist activity.

Molecular Models

The one invariable characteristic of the opioid peptides is a hydroxyl group on the N-terminal side chain of tyrosine, which is essential for opioid activity (*28,37*); interestingly, this side chain resembles the hydroxylated aromatic ring of the rigid alkaloid opiates. Shortly after the discovery of the enkephalins, structural models appeared that attempted to explain the interaction of the opioids with their receptors by simply superimposing the opioid on the ring structure and charged centers of the narcotic alkaloids (*28,60–62*), in spite of the X-ray diffraction analysis of enkephalin crystals which detailed the presence of a β-turn in the N-terminal region (*62*). Application of ^{1}H-NMR to study the topography of dermorphin and the deltorphins (*63–65*) revealed a strong propensity for these molecules to acquire a

paired dibasic residues. Similarly, both dynorphins A and B exist in a single prohormone along with β-neo-endorphin (*48*); however, even though [Leu[5]]enkephalin comprises the amino terminal pentapeptide in these peptides (*48*) and [Met[5]]enkephalin occurs in β-endorphin (*47*), these larger opioids are not further processed to the level of the enkephalin

molecule (*16*). The dermorphin precursor also contains multiple copies of the functional bioactive peptide within interesting homologous 35 amino acid repeats that, based on their primary sequence, would appear to form distinct α-helixes. However, the genomic information codes for the normal L-isomer of alanine, therefore indicating that the conversion to the
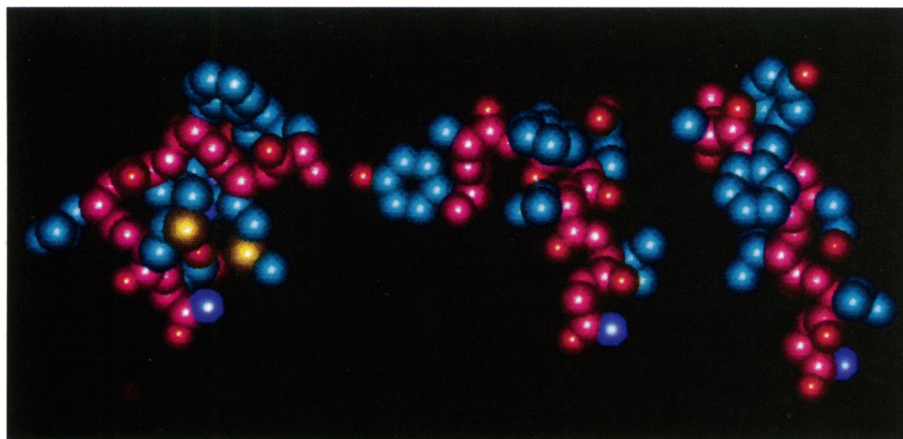
Figure 3. Molecular dynamics models of the deltorphins: (left), deltorphin A, deltorphin B, and (right) deltorphin C (see Table 1 for sequences) depicted with space-filling models. Magenta indicates the backbone, cyan the carbons of the side chains, purple the nitrogens (in histidine and amide groups), yellow the sulfur atoms (in the methionyl residues), and red the oxygen atoms.

type II′ β-turn in solution in the N-terminal tripeptide region with type I β-turns in the C-terminal domain as seen in deltorphin (65). These physicochemical data provided the rationale for the essential importance of the D-isomeric spatial orientation of the residue at position 2, and the backbone and side-chain dihedrals (torsion angles) of these peptides. Studies on the bioactivity (pharmacological preparations in vitro and production of analgesia in vivo) and receptor binding confirmed the absolute requirement of the D-enantiomer at position 2 since the stereo inversion around the α carbon to yield an L-isomer reduced opioid activity by several orders of magnitude (27,29).

The advent of computer modeling in combination with the intrinsic values for backbone and side-chain torsion angles derived from ^{1}H-NMR spectroscopy and NOESY (nuclear Overhauser effects spectroscopy) led to multiple model structures for enkephalin analogues (66,67). This method further provided the first direct correlation between the solution conformation of the flexible (non-constrained) deltorphins and receptor binding data (68). The solution conformation of the deltorphins, as seen in Figure 3, illustrates the topography of a peptide with the highest probability of binding in the receptor "pocket" at a specific receptor subsite; that is, deltorphin B interacts in a heterogeneous manner best described as fitting a two-site binding model (which may be equivalent to the δ_2 receptor subtype), while the binding of deltorphins A and C (δ_1 receptor ligands) are classically defined as interacting by means of a simple, bimolecular one-site model (73). With the emergence of a predictive means to propose active peptide conformations combined with a knowledge of low-energy conformations (although it should be pointed out

that the lowest energy conformation may not necessarily be biologically active), we systematically synthesized several hundred deltorphin analogues (and an equivalent number of dermorphin analogues) using solid-phase and solution methods (27,64,69–74). Thus, we were able to apply these amphibian opioid analogues as highly selective molecular probes to elucidate binding to selective receptors and to further differentiate between pharmacologically defined receptor subtypes (72,73).

Owing to the recent interest in the pharmacology of the deltorphin family of peptides, we highlight and briefly summarize the major chemical characteristics of these remarkable opioid peptides (Table 1), primarily determined in receptor binding studies with rat brain membranes (synaptosomes). 1) The properties of the side chain of the residue at position 4 enable the heptapeptide to differentiate between δ and μ receptors through influencing its affinity toward μ receptors; that is, whereas δ affinity remains relatively constant, the μ binding constants may fluctuate by two orders of magnitude. Moreover, residue 4 enables the peptide to discriminate between specific receptor subsites (based on statistically valid binding models) (72,73) that may reflect pharmacologically defined receptor subtypes (73). In deltorphin A, for instance, the imidazole (His^4) side chain is crucial for the expression of high δ affinity (27,69).

2) The ionizable anionic side chains play a minor role in δ receptor affinity. On the other hand, δ selectivity is nevertheless markedly enhanced because the acid function suppresses interaction with μ receptors (70,71); cations are detrimental for deltorphin-mediated binding. 3) Hydrophobicity, centered either at the fifth residue or in the composite nature of the lipophilicity of the C-terminal region, directly affects δ

affinity (71,73–76). [Similarly, the binding of dermorphin to μ receptors was enhanced by the presence of additional hydrophobic substituents (77).] 4) Deltorphin heptapeptide and abbreviated analogues containing an unnatural bicyclic-constrained amino acid in position 2 were surprisingly active (78–82). For example, the dipeptide Tyr-Tic-NH$_2$ represents the smallest peptide recognized by the δ opioid receptor and may represent the universal opioid "message" domain (82). Furthermore, the subtle change in chirality from L- to D-stereoisomer at position 2 in both di- (82,83) and heptapeptides dramatically switches the selectivity from one receptor type to the other. The systematic change to the D-stereochemistry of each individual residue in deltorphin A provided additional evidence that proper spatial orientation is necessary within the N-terminal pentapeptide region (27). (These combined observations represent one essential key to ferreting out the precise milieu, internal binding interactions, and structure of the receptor pocket.)

5) In our continuing exploration of replacement analogues using unnatural amino acids in the N-terminal sequence of deltorphins, we obtained peptides which exhibit either super δ receptor selectivity properties or have equivalent high affinities for both δ and μ receptors (73, unpublished observations). These unusual opioid peptides, as well as the di- tri-, and tetrapeptides (80–82), apparently produce a unique low-energy conformation in solution to fit unobtrusively (yet with great fidelity) into the δ receptor with exceptional selectivity (82,83, Bryant et al., unpublished observations). These data simultaneously invalidate opioid models in which opioid peptides were merely superimposed on the rigid rings of morphine (60–62).

Conclusions

The case for amphibian peptides as environmental opioids (or morphinomimetic substances) has singular importance as highly specific endogenous ligands acting on mammalian opioid receptors and is indeed quite compelling and substantiated by an enormous body of scientific evidence. To partially answer the questions posed (supra vide), at least two possible theories can account for the molecular mimicry of amphibian skin opioids for mammalian opioid receptors: 1) The opioid peptides could have evolved by convergent evolution to the endogenous peptides associated with vertebrate tissues. This idea stems from the concept of the "brain–gut–skin triangle" proposed by Erspamer (43,84) that states that peptides occurring in amphibian skin appear to exist in a similar, if not identical sequence, in mammalian

brain and gut; and 2) opioid peptides in skin secretions originally were used for different purposes by amphibians (and other forms of life) and acquired new functions over time (1). Thus, the opioids and their receptors have remained unchanged since the evolution of unicellular organisms due to the acquisition of a neuromodulator role. Of course, other theoretic alternatives exist to answer this tantalizing question, and only extensive experimentation with these remarkable compounds and their receptors will lead us toward more definitive conclusions.

The conservation of opioid peptides and receptors during evolution implies a "physiological function that confers a selective advantage on the organism" (85). Whether that function can be solely attributed to analgesia (85), however, remains an open question. During the early, formative period in evolution, opioids and opioid receptors presumably arose simultaneously (1,22) and were conserved thereafter in response to basic mechanisms of life: eat, survive, and reproduce. Excellent examples of this dictum can be found in the effect of opioids/opiates on protozoans: in Paramecium, the regulation of ion channels affects the direction of ciliary movement (23,24), which would propel it toward a food source or away from danger; in Tetrahymena, a reduction motility is noted (86) as well as the inhibition of phagocytosis (20), which would affect nutrient uptake. Thus, we might ask a further question: could opioid analgesia be a secondary manifestation of calcium regulation (or being undernourished for unicellular organisms; in other words, antagonism of molecular satiety) and with the evolution of multicellular organisms became associated with neurophysiological functions?

The study of amphibian opioid peptides as environmental chemicals that mimic endogenous mammalian substances affords us an excellent opportunity to selectively probe receptor molecules that are relevant in human health and disease. Furthermore, from their basic structure may eventually spring synthetic analogues to assist in the fight against the perennial problem of opiate addiction and many other equally valid health-related issues (alcoholism, neurological diseases, psychological abnormalities triggered by neurotransmitter imbalance and neurological dysfunctions, post-operative pain, memory loss in trauma victims and epileptic seizures, acute and chronic pain associated with terminal cancer, and prevention of graft rejection). In fact, many laboratories have initiated projects to further enhance the inherent in vitro stability and chemical properties of opioids to optimize their passage through the intractable blood–brain barrier

that would enable them to function as highly selective antagonists to relieve narcotic dependency and perhaps attenuate the psychopathological conditions of schizophrenia, depression (85), and Tourette's syndrome (87).

After millennia of being maligned, feared, and grossly misunderstood, frogs (and toads) might continue to bolster the well-being of human society—if they survive the detrimental onslaught of the effects of environmental pollutants (1,88). The fate of amphibians and humans are inexorably intertwined. The eons of amphibian existence and the brevity of humankind could be summed up by a quote from Shakespeare (89): "The oldest hath borne most: we that are young shall never see so much nor live so long."

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